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An Updated Guide to the Identification, Quantitation, and Imaging of the Crustacean Neuropeptidome

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Abstract

Crustaceans serve as a useful, simplified model for studying peptides and neuromodulation, as they contain numerous neuropeptide homologs to mammals and enable electrophysiological studies at the single-cell and neural circuit levels. Crustaceans contain well-defined neural networks, including the stomatogastric ganglion, oesophageal ganglion, commissural ganglia, and several neuropeptide-rich organs, such as the brain, pericardial organs, and sinus glands. As existing mass spectrometry (MS) methods are not readily amenable to neuropeptide studies, there is a great need for optimized sample preparation, data acquisition, and data analysis methods. Herein, we present a general workflow and detailed methods for MS-based neuropeptidomic analysis of crustacean tissue samples and circulating fluids. In conjunction with profiling, quantitation can also be performed with isotopic or isobaric labeling. Information regarding the localization patterns and changes of peptides can be studied via mass spectrometry imaging. Combining these sample preparation strategies and MS analytical techniques allows for a multi-faceted approach to obtaining deep knowledge of crustacean peptidergic signaling pathways.

Keywords

Crustacean; neuropeptides; peptidome; quantitation; MALDI mass spectrometry imaging; *in vivo* microdialysis; capillary electrophoresis; isotopic/isobaric labeling; *de novo* sequencing; DIA-MS

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[37.]The use of either a commercial or homemade column should be considered. Homemade packing lowers the cost of each column and allows for customization, which may improve peptide separation and thus coverage. However, low uniformity can cause inconsistency between columns in comparison to commercially available columns. Although, these columns can be much more expensive in comparison.

[38.]For peptides, the reversed-phase C18 column setup uses a gradient of water with 0.1% FA and ACN with 0.1% FA to elute the sample. Depending on the complexity of the sample, the time of gradient, highest percent ACN added, and instrumental parameters (*e.g.*, dynamic exclusion) will need to be adjusted.

[66.]The software tends to shift the selected area unpredictably after closing the window. Therefore, it is recommended to close and reopen the "View Plate" window after a selection has been made to ensure that the area of interest is still within the selection box.

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1. Introduction

Neuropeptides represent an expansive research area with tremendous potential for use in elucidation of human disease mechanisms and development of therapeutics. Fundamentally, neuropeptides are signaling molecules that originate from neurons, providing a snapshot of neuronal activity and processes. Fortunately, biological conservation of key neuropeptides has previously been established between humans and crustacean model organisms, providing an opportunity to study complex neuropeptide signaling processes in a simpler medium *(1)*. Crustacea are particularly appropriate as they have been documented to be rich in signaling molecules, including neuropeptides, in neurosecretory tissues, nervous systems, and the hemolymph, the fluid responsible for transport of neuropeptides and nutrients throughout, while maintaining a simple anatomical structure (Fig. 1)*(1)*. The stomatogastric nervous system is key to understanding critical signaling pathways, particularly with regards to the stomatogastric ganglion (STG), oesophageal ganglion (OG), and commissural ganglia (CoGs). Other tissues of interest include the brain (central nervous system), sinus glands (near the eyestalks), and pericardial organs (near the heart). There has also been significant work recently aimed at capturing the neuropeptide content within the hemolymph *(2)*.

Despite this, studying neuropeptides is accompanied with some fundamental hurdles, such as their susceptibility to degradation, low *in vivo* concentration, and high levels of sequence homology, with two distinct peptides differing by as little as a single amino acid residue, that complicate research efforts *(1)*. Additionally, neuropeptide cleavage from prohormones is difficult to predict, even with access to a complete genome or transcriptome, and neuropeptides undergo post-translational modification (PTM) that further complicate their identification. Many efforts have been devoted to developing methods to simplify investigation in this area. Mass spectrometry (MS) has emerged as a suitable means to identify and evaluate neuropeptides, due in part to its high mass resolution and sensitivity. Historically, there are numerous instances in which both electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques are utilized for neuropeptide investigations, typically driven by hypotheses related to specific applications *(3–5)*. Both ionization methods have distinct advantages, discussed herein.

We emphasize two primary types of ESI acquisition methods: data-dependent acquisition (DDA) and data-independent acquisition (DIA). DDA is significantly more established of the two methods and operates by selecting the top *n* most abundant precursor ions for MS/MS fragmentation. This is an efficient method with robust downstream data processing resources, but is inherently biased against ions of low abundance, minimizing the likelihood of acquisition of data corresponding to neuropeptides that are inherently low in concentration. DIA aims to minimize this biasing through a holistic acquisition method where all precursor ions within a particular *m/z* window are fragmented. While there are significant advantages to DIA methods, they produce highly convoluted spectra, complicating spectral identifications (Fig. 2). Despite these pros and cons of both methods, both DDA and DIA analyses have been successfully implemented with crustacean neuropeptides *(6–10)*. Herein we describe the preliminary considerations for both methods, as well as their respective data analysis workflows.

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Both database searching and *de novo* sequencing are common methods for neuropeptide identification from DDA ESI data. Database searching is a largely more robust method, but is limited to known neuropeptide sequences, whereas *de novo* sequencing requires no prior knowledge. While standalone software to conduct both search types exist, we primarily use PEAKS for our searches, as it can complete both search methods in a synergistic manner, where *de novo* search results inform the database search space (11, 12). For DIA data processing, two primary methods exist: spectral library-dependent searches and spectral library-free searching. As with before, library searches are considered to be more robust, but do require prior knowledge of the search space, as these methods typically use previously collected and annotated DDA spectra to identify neuropeptides from DIA spectra. Alternatively, library-free methods do not require prior knowledge, but do receive more scrutiny (13). Typically, these methods are performed by generating DDA pseudo-spectra from DIA spectra, and then processing with a standard DDA database searching platform. For our purposes, we conduct library-free analyses through use of DIA-Umpire (14), and we conduct library analyses through the use of PEAKS Online.

Mass spectrometry imaging (MSI), powered by MALDI-MS, is advantageous for the localization of neuropeptides within tissue sections. MSI provides an understanding of the spatial distribution of particular neuropeptides, enhanced with additional techniques including histological staining and traditional microscopy (15). For analysis of MSI data, there are a number of bottlenecks that impose challenges on data analysis pipelines, namely dataset size limitations, that are further prohibitory due to software costs and experience with programming required (16). In this chapter, we describe methods for processing imaging datasets with ImageQuest (Thermo), flexAnalysis (Bruker), SCiLS lab (Bruker), and MSiReader (17, 18) (open source). Additionally, 3D imaging generation is often conducted in ImageJ (19).

Previously, we presented a comprehensive description of methods for detection, identification, and quantitation of crustacean neuropeptides (20). Since this time, the field has seen tremendous growth in vital areas. With sample preparation, the successful application of enrichment and derivatization methods has been achieved in neuropeptide extracts (21–24). Many quantitation methods for both label-free quantitation and labeled quantitation have also been developed (21, 23–26). With regards to ESI-based MS, notable efforts have been made to apply and optimize DIA strategies (7–10). Alternatively, there have also been substantial advancements in MSI, a necessity for gaining an understanding of the spatial distribution of neuropeptides (15, 27–29). From a bioinformatics perspective, the past 5 years has seen substantial progress, as we have been able to combine a rich history of MS software solutions with the particular attributes of neuropeptides to improve the ability to accurately identify and quantify neuropeptides (30, 31). These overall advances in technology have allowed for specialized studies to be conducted in exciting areas including feeding (10, 32, 33), copper toxicity exposure (23), pH stress (21, 34) and hypoxia (18, 24, 35). Efforts have also been devoted to developing specific enrichment strategies for unique neuropeptide modifications and isoforms, such as glyconeuropeptides, which are particularly relevant to human health (22). It is our hope that this updated primer of best practices in MS analysis of neuropeptides will inspire further advances within the field. Herein, we describe the fundamental methods required for neuropeptide analysis, as well

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as emerging methods and techniques, including new sample preparation methods, DIA strategies, glyconeuropeptide characterization, and advances in MSI.

2. Materials

2.1 Chemicals and Equipment

2.1.1 Chemicals

- Ultrapure water (HPLC grade), used for all the solutions listed in all experiments. Optima™ LC/MS Grade water was used for all samples intended for mass spectrometers.
- Methanol (MeOH)
- Acetonitrile (ACN)
- Formic acid (FA)
- Crab saline (440 mM NaCl, 11 mM KCl, 26 mM $MgCl_2$, 13 mM $CaCl_2$, 11 mM TRIS, 5 mM maleic acid, adjusted to pH 7.45 with HCl or NaOH. Stored at 4 °C)
- Acidified methanol (90:9:1 (v: v: v) MeOH: H_2O: glacial acetic acid)
- 10 mM Ammonium formate (AF)
- Isotopic formaldehyde solution (1% v/v)
- 30 mM Borane pyridine solution
- 100 mM Ammonium bicarbonate solution
- DiLeu tags *(26)*
- DiLeu labeling buffer (50/50 ACN/0.5M triethylammonium bicarbonate, TEAB)
- Anhydrous *N*,*N*-dimethylformamide (DMF)
- N-metheylmorpholine (NMM)
- 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM BF4)
- Activation solution (15.5 mg DMTMM, 495 μL dry DMF, 5 μL NMM)
- 5% hydroxylamine in H_2O
- SCX loading buffer (0.1% FA, 20% ACN)
- SCX elution buffer (0.4M ammonium formate, 20% ACN)
- Trifluoroacetic acid (TFA)
- Gelatin (100 mg/mL dissolved in H_2O)
- Background electrolyte solution (BGE) (50 mM AcOH buffer solution at pH 4.5)
- Matrix-assisted laser desorption/ionization (MALDI) matrix

- – 2,5-dihydroxybenzoic acid (DHB) dissolved in 50:50 MeOH: H_2O with 0.1% FA (150 mg/mL for spots; 40 mg/mL for imaging)
- – α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in 50:50 ACN: H_2O with 0.1% FA (10 mg/mL for spots; 5 mg/mL for imaging)

- Acetic acid (AcOH)
- 10% neutral buffered formalin (NBF): 94:4:1:0.5:0.5 (v/v/v/w/w) H_2O: formaldehyde: MeOH: sodium phosphate dibasic: sodium phosphate monobasic

2.1.2 Equipment

- Glass manual homogenizer (*e.g.* Wheaten 1 mL Tissue Grinder, Tenbroeck)
- Bath sonicator
- Centrifuge capable of up to 16,000 g and able to fit microfuge tubes and microfilters (*e.g.* Eppendorf 5424 R)
- Vacuum centrifuge/concentrator, such as a SpeedVac (*e.g.* Savant SVC100)
- Solvent syringe pump
- Automated sample collector with temperature control
- Vortex
- Sprayer for MALDI imaging (*e.g.* HTX Technologies)
- Cryostat

2.1.3 Other materials

- Tweezers
- Teflon pestle (*e.g.* Wheaten 1 mL Tissue Grinder, Tapered Pestle)
- Metal dishpan
- 25 g needle
- 1 mL syringe
- 3 kDa or 10 kDa Molecular Weight Cutoff (MWCO) device (*e.g.* Amicon Ultra)
- Reversed-phase C18-packed pipette tips (*e.g.* Omix 100 μL Tips or Millipore P10 ZipTips)
- Pierce Peptide Assay Kit
- Strong cation exchange (SCX) resin prepacked tips
- HILIC beads (*e.g.* PolyHYDROXYETHYL A from PolyLC INC.)
- TopTips
- Cotton ball
- MALDI plate

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- Plastic tissue embedding base mold
- Razor blade
- ITO (indium-tin-oxide) coated glass slide

2.2 Instrumentation and Software

Prior to mass spectrometry, off-line separation can be performed to preprocess the samples (we use a Waters Alliance 2695 Separations Module). We also used capillary electrophoresis for separating low volume samples (we use a Hewlett Packard 3D CE). For quantitation and profiling, a Hybrid Quadrupole-Orbitrap Mass Spectrometer (we use a Thermo Q-Exactive and Orbitrap Fusion Lumos Tribrid mass spectrometer) with a nano-electrospray ionization (ESI) source can be coupled to a UPLC system (we use a Waters nanoACQUITY and Dionex Thermo Ultimate 3000) for separation of peptides with MS and MS/MS analysis for identification and quantitation. For imaging and complementary profiling, we use a hybrid MALDI- Ion Trap-Orbitrap Mass Spectrometer (we use a Thermo MALDI-LTQ-Orbitrap XL) and MALDI-TOF/TOF mass spectrometer (we use a Bruker rapifleX). We also used capillary electrophoresis as a hyphenated front-end separation platform in our MALDI experiments. Other mass spectrometers with a MALDI source and an ESI source may also be used. The associated vendor software of each instrument can be used for data analysis and image processing. For MS/MS identification, *de novo* sequencing software can be used, such as PEAKS *(*12*)*, MaxQuant *(*36*)*, or PepNovo *(*37*)*. Prior to *de novo* sequencing, PRESnovo can be used *(*30*)*. Following *de novo* sequencing, HyPep can be used to tune results for neuropeptides *(*31*)*. For DIA workflows, spectral library searches can also be conducted in PEAKS Online, and library-free searches can be completed using DIA-Umpire followed by PEAKS Studio. Additional image processing can be performed using freely available software, such as MSiReader *(*17*)* and Image J *(*38*)*.

3 Methods

3.1 Identification and Quantitation (Fig. 3)

1. Sample Collection and Extraction
   - **A.** Tissues
     - **i.** Collection
       1. Collect the tissue of interest through dissection *(*39*)*.
       2. Place tissues in a 0.6 mL microfuge tube with 10 to 20 μL of acidified methanol.
       3. Store at −80° C until ready to use (*see* Note 1).
     - **ii.** Extraction

[1]While storage at −80 °C minimizes postmortem degradation, tissues should only be stored for up to six months before use. There are several ways to decrease postmortem degradation for both tissues and hemolymph, which would extend their storage life. For tissues, the use of a Denator heat stabilizer system or boiling of the tissues for heat stabilization has been shown to be effective *(*42*)*. For hemolymph, the addition of protease inhibitor cocktail or EDTA can reduce protease activity *(*43*)*.

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1. Add 100 μL of acidified methanol per tissue to a glass manual homogenizer (*see* Notes 2, 3, 4, and 5).
2. Move the tissues to the homogenizer using tweezers. Transfer the storage solution as well.
3. Homogenize the tissue until no large particles are visible. Transfer the liquid to a clean 1.5 mL microfuge tube.
4. Add 100 μL of acidified methanol to the homogenizer, breaking up any residual particles. Transfer to same microfuge tube. Repeat once more.
5. Sonicate mixture for 10 min.
6. Centrifuge mixture for 20 min at >16,000 g.
7. Transfer the supernatant to a new 1.5 mL tube.
8. Rinse the pellet with 100 μL of acidified methanol and break it up with a Teflon pestle.
9. Repeat steps 5-7. Discard the pellet.
10. Dry down supernatant in a vacuum centrifuge on medium heat (*see* Note 6).

B. Hemolymph

i. Collection

1. Place the crab on ice for 5-10 min. Afterwards, place it in a metal dishpan on its back with its tail pointing towards you.
2. Prepare a 25 g needle by connecting it to a 1 mL plastic syringe.
3. Place the needle into an exposed leg joint of the crab.

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2. Several different extraction solvents have been tested for their ability to extract neuropeptides from tissues, including acidified organic solvents *(44)*.

3. While a manual homogenizer has been shown to be effective for small crustacean tissues, an electronic sonicator may be needed to fully break down the tissue walls of large organs. These should be used with the tissues on ice to help with heat dissipation.

4. When deciding how many tissues to pool, it is important to balance biological variance with the number of tissues required for the study. In most stress-related studies, at least 3 animals' tissues are pooled for each biological replicate.

5. Direct profiling is an alternative strategy to extraction. The tissue of interest can be placed on the stainless-steel spotting plate, with matrix being spotted directly onto the tissue area of interest. This method has become phased out with the development of new imaging techniques (see "Localization using Imaging" section in this book chapter).

6. Many crustacean peptides vary widely in their molecular masses. For example, crustacean neuropeptides can range from 0.5 to 9 kDa. With many orbitrap-based instruments, it is difficult to analyze the larger peptides without taking a bottom-up approach. This means some sort of enzymatic digestion prior to analysis may be required to get a full picture of a crustacean peptidome. For crustacean peptides, the digestion is usually done on the initial extract. After reducing all disulfide bonds with dithiothreitol and alkylating with iodoacetamide, an enzyme (*e.g.*, trypsin) is added at a 25-50:1 peptide: trypsin ratio. Peptide content can be determined using the bicinchoninic acid assay or a similar assay.

4. Pull up on the syringe to create a vacuum. Hemolymph should come out immediately. If not, wiggle the needle at different angles until the liquid is withdrawn.

5. Add an equal amount of acidified methanol to the collected hemolymph in a 1.5- or 2-mL microfuge tube (*see* Note 7). A protein precipitate will be produced.

6. Store at −80 °C till ready to use (*see* Note 1).

ii. Extraction

1. Using the Teflon pestle, break up the precipitate in the microfuge tube to produce a homogenous solution.

2. Sonicate tube for 10 min.

3. Centrifuge mixture for 20 min at >16,000 g.

4. Transfer the supernatant to a new 2 mL microfuge tube.

5. Rinse the pellet with 500 μL of acidified methanol and break it up with a Teflon pestle.

6. Repeat steps 2-5. Discard the pellet.

7. Dry down supernatant in vacuum centrifuge on medium heat (*see* Note 6 and 8).

iii. Microfiltration

1. Add 200 μL of 0.1 M NaOH solution to the 3 kDa or 10 kDa MWCO device (*see* Note 9). Centrifuge at 14,000 g for 5 min.

2. Rinse MWCO device with 500 μL of 50:50 water:methanol. Centrifuge for 10 min at 14,000 g.

3. Dissolve sample in 500 μL of 30:70 water:methanol, vortex, and sonicate for 10 min.

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[7]. Each crab has a limited amount of hemolymph stored in its body. For example, *Callinectus sapidus* has approximately 5 ml. After removal, hemolymph is restored over a period of time. Thus, the amount of hemolymph removed depends on the species of crab and whether it is to survive afterwards. Replacing the volume of removed hemolymph with an equal volume of crab saline can increase the crab's chance of survival.

[8]. After drying down any hemolymph samples (initially to fully processed), they should be resuspended prior to storage at −80 °C to ensure full dissolution.

[9]. Depending on the size of the peptides of interest, two versions of MWCO filters are available: 3- or 10-kDa. 10 kDa is normally used to purify a wide mass range of crustacean peptides (*see* Note 6). After digestion, 3 K MWCO filters can be used to simplify the sample and allow for a targeted analysis.

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4. Add the sample to the MWCO device and run it through the membrane by centrifuging for 30 min at 14,000 g. Save the flow-through from this step by placing it in a new collection tube, (*see* Note 10).

5. Rinse the membrane with 100 μL of 30:70 water methanol. Collect the flow-through and add it to the tube from the previous step.

6. Dry down the flow-through in vacuum centrifuge on medium heat.

C. Microdialysis (*see* Notes 11 and 12)

i. Collection

1. Rinse probe with water and then crab saline using a 3 mL plastic syringe and a syringe pump set to 0.5 μL per min.

2. Surgically implant the probe into the animal directly above its heart *(*40*)*. Fig. 1 indicates the location of the heart in *Cancer borealis*.

3. Allow the animal to recover for 24- to 48-hours (*see* Note 13).

4. Collect samples at desired time points. Collection can be done manually with a 0.6 mL centrifuge tube on ice or using an automated sample collector set at 4°C (*see* Notes 14, 15, and 16).

5. Add enough formic acid to reach a total concentration of 0.1% (v/v).

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10. Sometimes, the MWCO filters become clogged with proteins that (a) were not fully pelleted in the extraction step or (b) were not dissolved in the MeOH:H_2O mixture that was added to the extract. To decrease the chance of this happening, (a) centrifuge the extract at >16,000 g for 15-20 min before putting the supernatant through the filter, (b) separate the extract into two fractions with two MWCO filters, or (c) centrifuge the extract through the filter for a longer period of time (45+ min).

11. There are several types of commercial probes that can be used for microdialysis. Alternatively, homemade probes can be implemented at a much lower cost. Whether purchasing or making probes, there are several factors worth considering, including membrane material, the molecular weight cutoff (the expected MWCO is approximately a third of the reported MWCO), area of membrane, and use of polymer coatings *(*45*)*.

12. The microdialysis technique tends to have low recovery *in vivo*. The recovery can be enhanced through the use of affinity agents *(*46*)*

13. It is important to closely monitor the crab while it recovers from surgery, as the majority of post-surgery complications (*i.e.*, crab dying, pulling out probe, clogging probe) typically occur within the first 24 h.

14. Collection windows are typically within the range of 30 min to 2 h. This is dependent on the necessary temporal resolution and recovery of sample. Shorter collection durations provide higher resolutions but yield lower sample volumes. Collection times of several minutes have been shown feasible, often using online microdialysis collection coupled to ESI. However, this method also comes with drawbacks *(*47*)*.

15. The optimum infusion rate for microdialysis is a tradeoff between relative recovery and absolute recovery. Lower flow rates allow for more diffusion of sample through the membrane, but result in less sample volume, while higher flow rates enable the collection of more sample overall but run the risk of disturbing the crab. We have found the ideal flow rate to be 0.5 μL/min.

16. When collecting samples, it is important to account for the dead volume between the tip of the probe and the end of the tubing (and collection needle, if one is being used). The delay from sample diffusing into the probe to reaching the end of the tubing could be one hour or greater, depending on the length of tubing and infusion rate.

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6. Store samples at −80 °C if they are not being analyzed immediately. However, samples should be used as soon as possible to avoid degradation (*see* Note 17).

2. Desalting

   I. Dissolve each sample in 0.1% FA (10 μL for tissues, 200 μL for hemolymph) by vortexing and sonicating for 10 min (microdialysis samples do not need to be redissolved.). If the pH is greater than 3, add small amounts of diluted FA (*e.g.* 10%) until the pH is below 3.

   II. Using the appropriately sized reversed-phase C18 pipette tip and respective volume (*see* Note 18) (10 μL for tissues and microdialysate, 100 μL for hemolymph), flush the packing material with pure ACN at least 3 times, discard the ACN.

   III. Equilibrate the packing material with 0.1% FA 3 times and discard the 0.1% FA.

   IV. Bind the sample of interest to the packing material by flushing it slowly with dissolved sample at least 15 times.

   V. Wash the packing material with 0.1% FA 3 times to wash away salts and other unbound contaminates. Add the first wash to its own tube or to the original sample vial as a precaution in the event that nothing binds to the packing material.

   VI. Elute the sample into a 0.6 mL tube by flushing 10 μL of 50:50 ACN: H_2O up and down through the packing material 10 times.

   VII. Dry down the eluate in a vacuum centrifuge on medium heat.

3. Off-line Fractionation (*see* Notes 19 to 21)

   I. After sample extraction, reconstitute the samples in Optima™ LC/MS Grade H_2O with 10 mM AF (pH=10).

   II. Fractionate samples, a maximum of 100 μL of sample at a time (we use a Waters Alliance 2695 Separations Module which has an 8 μL dead volume).

   III. Fractionate samples using a Kinetex® Core-Shell EVO 5um C18 column at 0.2 mL/min with a column temperature at 30 °C (*see* Note 20).

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17. Microdialysis samples have been shown to degrade rapidly, even within several hours. Therefore, samples should be analyzed as soon as possible after collection. If longer storage time is necessary, there are several methods for improving the lifetime of the samples (47, 48).

18. Many different varieties of separations can be done to increase peptide purity, including strong cation exchange (SCX) and C18 pipette tips (*e.g.*, TopTips and ZipTips, respectively). Success has also been found in increasing peptide coverage in fractionation using C18 pipette tips or an HPLC system (49).

19. We use 10mM FA in water as mobile phase A, and 10mM FA in with 90% ACN as mobile phase B.

21. The detailed method and fractionation gradient can be found in ref. (50)

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IV. In the instrumental interface, set sequence name, volume and select the optimum method for fractionation (*see* Note 21).

V. Run sequence and collect the fractionated samples.

VI. Dry down the fractions in a vacuum centrifuge on medium heat.

VII. The sample is then ready to be analyzed by mass spectrometer.

4. Quantitative Labeling

A. Isotopic Labelling through Reductive Dimethylation

i. Dissolve each of the samples (*e.g*stressed and control) in 10 μL H_2O by vortexing and then sonicating for 10 min.

ii. Add 10 μL of isotopic formaldehyde to its designated channel (*see* Notes 22 and 23).

iii. Add 10 μL of 30 mM borane pyridine solution to each microfuge tube.

iv. Place the tubes in a 37 °C water bath for 15 min to allow complete labeling.

v. Quench the reaction with 10 μL of 100 mM ammonium bicarbonate solution.

vi. Mix equal amounts of each channel into a 0.6 mL tube. Do this for each set of channels.

vii. Dry down mixture in a vacuum centrifuge on medium heat and sample is ready to be analyzed using a mass spectrometer.

B. Isobaric labelling using DiLeu tag (*see* Note 24).

i. Quantify peptide sample concentration (we use a Pierce Peptide Assay Kit and follow the manufacturer's procedure).

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[20]. Before the run, turn on the UV-Vis lamp and condition the column for at least 10 mins. The optimum column pressure needs to be achieved before running (for our column, we need ~1200 psi).

[22]. Formaldehyde labeling is commonly used to produce 2 or 3 different isotopic forms that differ in mass (51–53), although up to 5 different isotopic forms can be generated with different combinations of heavy and light formaldehyde and reducing agent (54). Other labeling methods exist, such as isotopic *N*, *N*-dimethyl leucine (iDiLeu), which boasts 5-plex labeling capabilities, which allows for relative or absolute quantitation by an in-solution calibration curve (55). Absolute quantitation of a single peptide can also be done by adding an isotopically enriched (*e.g.*, deuterated) version of the peptide to the original extract of the sample.

[23]. For crustacean tissues, formaldehyde labeling has been shown to be extremely effective due to its quick and complete labeling of peptides. While MS-based quantitation strategies are simple, they have limitations, especially when analyzing more than 5 samples or samples with high spectral complexity. On the other hand, MS/MS-based quantitation strategies, such as iTRAQ, TMT, or *N*,*N*-dimethyl leucine (DiLeu), allow for higher multiplexing (up to 21 sample comparisons at once have been demonstrated) with lower MS spectral complexity (56, 57). Unfortunately, quantitative depth may suffer because, to become quantified, the peptide needs to be selected for MS/MS. For DIA analyses, there has been much discussion surrounding the impact of instrumentation setting selection, such as with regards to collision energy and automatic gain control. See ref. (13) for a discussion of the fundamentals of each of these parameters, as well as our recent work ref. (58), where we work to determine the optimal parameters for DIA methods for neuropeptides.

[24]. DiLeu tags are synthesized in house and the procedure can be found in ref. (26). The synthesizing procedure is not discussed in this protocol.

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- **ii.** After relative quantitation, split the samples into 25 μL aliquots and dry down the sample with vacuum centrifuge on medium heat. Store at −80 °C until labeling.
- **iii.** Prepare activation solution and 1 mg of dry DiLeu tag, vortex at RT for 30 min (*see* Note 25).
- **iv.** Resuspend the 25 μg sample aliquots from above in 53.57 μL labeling buffer.
- **v.** Add 500 μg of the activated DiLeu tag from above to the resuspended peptide sample and vortex at RT for 1 h (*see* Note 26).
- **vi.** Quench the reaction with 4.7 μL of 5% hydroxylamine to a final concentration of 0.25%.
- **vii.** Pool differentially labeled samples together (1:1 ratio) and dry down the pooled sample in a vacuum centrifuge on medium heat.
- **viii.** Remove excess labeling reagent using SCX spin tips (we use polyLC SCX TopTips) according to the manufacturer's protocol (*see* Note 27).
- **ix.** Dry the sample down in a vacuum centrifuge on medium heat and store at −80 °C until further instrumental analysis.
- **x.** Desalt samples according to above procedures, using a desalting tip with larger binding capacity (we use Omix C18 Tips, 10-100 μL) to accommodate greater sample quantity.
- **xi.** Dry the sample down in a vacuum centrifuge on medium heat and store at −80 °C until further instrumental analysis.

5. Glycopeptide enrichment through HILIC (*see* Note 28).

- **A.** Mix 12 mg of the HILIC beads with 200 μL of 1% TFA to create a slurry (beads:peptides mass ratio = 30:1).
- **B.** Load HILIC slurry on top of 3 mg of cotton wool in the TopTip.
- **C.** Resuspend the peptide sample (either tissue sample or hemolymph sample) in 300 μL 95% ACN, 1% TFA, load onto the spin tip, and centrifuge (4 °C, 1000 g, 5 min).
- **D.** Collect the flowthrough and reload it. Repeat this step 5 times.

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25. This activation procedure can produce up to 700 μg of activated DiLeu tag.
26. The specific volume described here is to label a set quantity of neuropeptides. We use a 20:1 label:peptide ratio.
27. Briefly, resuspend the sample in 100 μL SCX loading buffer. Wash and equilibrate the resin in the spin tips and load the sample. After loading, wash the tip with 100 μL loading buffer at least five times, and elute the cleaned-up sample with 100 μL SCX elution buffer three times.
28. A spin-tip cotton HILIC enrichment method is used in this case and detailed procedure can be found in ref. (*59*).

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E. Wash the spin tip 5 times use 300 μL 95% ACN, 1% TFA solution.

F. Use a new collection tube and elute the peptide sample with 300 μL 80% ACN, 5% FA by centrifuging (4 °C, 2000 g, 3 min).

G. In the same collection tube, elute the sample again with 30% ACN 5% FA by centrifuging (4 °C, 2000 g, 3 min).

H. Dry the sample down in a vacuum centrifuge on medium heat and store at −80°C until further instrumental analysis.

6. Tissue Analysis and Data Acquisition

A. MALDI-MS

i. Capillary Electrophoresis (*see* Note 29).

1. Inject 1 μL of the peptide sample through the capillary using pressure injection (12% of the capillary volume).

2. Move the inlet capillary into a vial of BGE (50 mM AcOH buffer solution at pH 4.5) and apply −20 kV for 30 min for the CE separation.

3. Place the chipped part (*see* Note 29) at the outlet of the capillary in a reservoir filled with BGE solution and leave the tip of the outlet outside of the reservoir.

4. For spotting experiment, place the outlet tip of the capillary on one spot of the MALDI plate for 30 s for sample collection and move to the next spot. For continuous sample collection, attach the MALDI plate to a syringe pump and set the platform to move at 4.2 mm/min (*see* Note 29).

5. Apply 0.2 μL of DHB matrix manually for spot experiment or use TM sprayer to coat the plate (12 passes of DHB, 80 °C, 30 s drying time between passes).

6. Analyze the sample on MALDI plate with MALDI-Orbitrap.

ii. Direct Spot Analysis

1. Dissolve desalted samples in 5 μL of 0.1% FA for each tissue used vortexing and sonicating for 10 min (*see* Note 30).

---

[29]. The design and instruction of CE-MALDI can be found in ref. (*28*) and will not be discussed here in detail.
[30]. Prior to spotting with matrix, an offline separation can be done, for example with capillary electrophoresis or high-pressure liquid chromatography (HPLC). Success has been seen for separating tryptic peptides with both high-pH and strong-cation-exchange chromatography (*50, 60*).

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2. In a separate 0.6 mL tube, mix equal amounts of matrix (*see* Note 31) and sample (*see* Note 32) solutions, vortexing them to ensure complete mixing.

3. Take 1 μL from the tube using a pipette and spot it on the MALDI stainless steel plate. Rub the tip of the pipette along the edges of the spot circle to make sure the whole circle is filled.

4. Allow the matrix to crystalize fully before placing in the instrument.

iii. Analysis on MALDI-MS

1. If necessary, attach a backing plate to the MALDI stainless steel spot plate (Thermo MALDI-LTQ-Orbitrap XL and Bruker rapifleX require this), and insert the plate set into the instrument by placing the plate in the correct position and pressing the appropriate button on the vendor's tune page (*e.g.*, the "Insert MALDI sample plate" button on the "MALDI source" window of the Thermo interface).

2. Once the plate has been inserted, confirm the spot in which you placed your sample and shoot the spot with an appropriate laser energy to obtain a signal intensity (*see* Note 33).

3. For Thermo MALDI-LTQ-Orbitrap XL

   a. Open Thermo Xcalibur Sequence Setup and create a new sequence (or use alternative vendor's software with regard to its specifications).

   b. Fill the sequence, including the title, path (for saving the data), instrumental method (*see* Note 34), and spot position (*see* Note 35).

---

31. Several matrices work well for crustacean peptide analysis, including DHB and CHCA. While DHB extracts peptides well, CHCA, known for being a "universal matrix," tends to provide a more homogenous layer. The laser energy used is also determined by the matrix type. For CHCA, lower energy tends to be used, and higher laser energy is applied for DHB matrix.

32. Several spotting techniques exist, including the suggested premixing, alternatives are sandwiching, or individual mixing on plate prior to recrystallization.

33. Depending on the instrument, lifetime of the laser, instrumental setup, and the matrix of choice, the laser energy used will need to be optimized for each sample.

34. In order to acquire more confident identifications or perform *de novo* sequencing, tandem MS is necessary. For MALDI instruments, collision-induced dissociation and high-energy collisional dissociation are the main commercially-available options. Unfortunately, MALDI ionization mainly produces singly-charged ions, which lead to poor fragmentation. This means that most identifications for MALDI are done through accurate mass matching. ESI provides much higher-quality fragmentation spectra, meaning that tandem MS can be used for high-confidence identification and discovery of novel peptides. As another fragmentation option, electron transfer dissociation, which is better for post-translational modification analysis, is now being more readily available. Even with high quality tandem MS spectra, *de novo* sequencing of peptides can be challenging. The use of chemical derivatization

c. Start automated or manual acquisition of every spot of the sequence (in Xcalibur: Select each line to be run and press the "Run Sequence" button).

4. For Bruker rapifleX

a. Set the laser energy based on the matrix selection (*see* Note 31) and acquire the mass spectrometry manually for the desired spots.

b. Save the acquired spectra through the flexControl interface.

B. ESI-MS (*see* Notes 36 to 39)

i. Sample Prep

1. Dissolve samples (approximately 5 µg on average) in 5 µL of 0.1% FA per tissue used or per 0.25 mL hemolymph by vortexing and then sonicating for 10 min.

2. Transfer the volume into a LC vial with a screw top septum (we use Waters Total Recovery Vials), making sure to get all the volume to the bottom with no bubbles.

ii. MS analysis (we use a Q-Exactive Orbitrap and Orbitrap Fusion Lumos Tribrid Mass Spectrometer, Thermo)

1. Place the LC vial into the chilled sample compartment of the LC system (we use a Waters NanoACQUITY). Make note of the tray number and tray position in which the samples were placed.

2. Make sure the column system has been equilibrated to the starting conditions of the gradient you will be using (*see* Note 36).

3. On the instrument profile, set up the sequence as described above (*see* Note 34).

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with a non-isotopic version of formaldehyde (see Note 22) has allowed for more complete coverage when *de novo* sequencing putative peptides (*61*). Other methods, such as thiol reduction and alkylation have also been used to achieve high-resolution sequencing of larger, disulfide-bonded crustacean peptides (*34*).

[35]. Xcalibur software only recognizes plates as having 96 wells, so it will only allow you pick spots that are within that plate size, even if you are using a 384-well plate.

[36]. Before LC separation, equilibrate the column with high, medium, and low concentrations of organic mobile phase B, with each for 20 mins, and wait the column pressure to stabilize at around 7000 psi or 500 bar. Besides classic LC separation, several other complementary options exist. Capillary electrophoresis is compatible offline for both MALDI and ESI analysis for enhanced separation of crustacean neuropeptides (*62–64*). Ion mobility has also been shown to be effective at separating D/L-epimeric crustacean peptides (*65*).

[39]. When beginning any LC-MS experiment, a quality control (QC) sample should be run to determine if the instrument is working optimally. Also, blanks should be run between each new sample type to decrease sample overlap.

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4. Select each sample in the sequence (*see* Note 39) to be run (in Xcalibur: press the "Run Sequence" button).

7. Data Analysis

A. MALDI-MS

i. For Thermo MALDI-LTQ-Orbitrap XL

1. Average all the MS spectra collected by left clicking and dragging across the chromatogram.

2. Copy the peak list from your vendor's software using the exact masses (Xcalibur: by right-clicking on the spectrum, selecting "Export" and "Clipboard (exact mass))."

3. Paste data into a blank spreadsheet file (we use Excel, Microsoft).

4. Compare the masses found to the in-house database through accurate mass matching (unlabeled or labeled) (*see* Notes 40 and 41). See Fig. 4 for an example spectrum,

ii. For Bruker rapifleX

a. Open flexAnalysis and select the saved mass spectrometry files.

b. Perform baseline subtraction and smoothing as desired.

c. Use "Find peak list" to obtain the list of m/z for the peaks observed.

d. Export the peak list to Microsoft Excel and compare the masses found to the in-house database through accurate mass matching (unlabeled or labeled).

B. ESI-MS (Fig. 5)

i. PRESnovo (*30*)

1. Import motif database and precursor file.

2. Run analysis.

ii. PEAKS Studio Search (DDA or library-free DIA) (*11, 12*)

1. Load the raw files by creating a new project.

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[40] For orbitrap instruments, the standard mass tolerance is +/− 5 ppm.
[41] This can be done manually or with a simple peak-picking program.

2. Select the file type, experiment type (DDA or DIA, *see* Note 42), instrument type, and enzyme selection (for endogenous peptidomics, we recommend "No enzyme").

3. Select desired workflow (Database search or Spectral library search)

4. Process the samples (using the "PEAKS search" button), indicating any enzyme used, any PTMs expected (*see* Note 43), and choosing the in-house database. *De novo* sequencing will be done with the same parameters (*see* Note 44).

5. Once the search is complete, quantitative information can be mined (*see* Note 45) in PEAKS by using the "Quantification" icon (*see* Note 46).

iii. HyPep (DDA, *see* Note 47) (31)

1. Launch HyPep via the command line interface.

2. Select search constraints (*see* Note 48).

3. Select all input files, including database, *de novo* sequencing results, and spectrum files.

4. Run analysis.

iv. PEAKS Online Spectral Library Building and Searching (DIA, *see* Note 49) (41)

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42. For library-free DIA, experiment type should be set as "DDA", because DIA-Umpire converted the DIA spectra to DDA pseudo-spectra.

43. Common post-translational modifications (PTMs) for crustacean neuropeptides include amidation and dehydration. If any labeling is done, it is important to include the tag or modification during the search (*e.g.* dimethylation).

44. PEAKS software package works by first *de novo* sequencing all of the raw data, which will then be matched to the provided database of precursor masses and proposed sequences. It is important to note that, while other databases come from sequenced genomes, the crustacean neuropeptide database has been developed in-house, as there is no genomic database for crustaceans. When looking at peptides with no digestion, PEAKS shows the peptide searched as a "protein" in the program. The "peptides" section is the individual peptides that make up all the crustacean peptides, which can include cleaved or degraded derivatives. Anything that doesn't match is placed in a "*de novo* only" tab, which can provide possible novel peptide groups. For neuropeptides, these "*de novo* only" peptides are compared to current family sequences, from which certain sequence themes can be found to identify novel neuropeptides. Confident identification can then be done by synthesizing standards to confirm MS/MS and LC retention patterns.

45. Several software packages are capable of performing *de novo* sequencing, database searching, and peak peaking for quantitation besides PEAKS (*e.g.*, Proteome Discoverer).

46. Both MS and MS/MS-based quantitation can be done, depending on the labeling used in the quantitation step. Label-free quantitation is also possible.

47. Prior to running HyPep, it is expected that the .raw spectrum have been processed by PEAKS (*de novo* sequencing) (11), TopFD (spectral deconvolution) (66), and RawConverter (file format conversion) (67). It is also anticipated that a directory of theoretical spectra for each database entry has been previously compiled, for example from ProteinProspector (https://prospector.ucsf.edu/prospector/mshome.htm).

48. Selection choices are made from preliminary data analyses. For FDR algorithm, shuffle is regarded as superior. Selection choices include: FDR % Threshold (we use 1%), sliding window size (3), max precursor charge (+8), max fragment charge (+4), precursor error cutoff (20 ppm), fragment error cutoff (0.02 Da), promex score cutoff (−10), and # matching loops (5), with optimal values in parentheses.

49. PEAKS Studio is also capable of spectral library searching, but we have had notably more success completing searches in PEAKS Online.

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1. Input DDA spectra for library building, analyzing as above.
2. Following data analysis, select to generate and export spectral library from DDA data.
3. Load DIA spectra, selecting to search against the established spectral library.

v. DIA-Umpire (library-free DIA, *see* Note 50) *(*14*)*

1. Convert all input files to .mzXML format (*see* Note 51).
2. Load parameter file (*see* Note 52).
3. Run analysis.

3.2 Localization by Mass Spectrometric Imaging (Fig. 6)

1. Sample Preparation

A. Small or thin tissue (*e.g.* PO, STNS, STG as in Fig. 1)

i. Collect the tissue of interest by means of dissection *(*39*)*, being mindful of tissue orientation (*see* Note 53).

ii. Hold onto tissue with tweezers and briefly submerge it in a microfuge tube of water to desalt (*see* Note 54).

iii. Place the clean tissue directly on a glass slide. Use a marker to label the back of the glass slide with the orientation of the tissue (*i.e.* which area is closest to the head, tail, etc.). Stretch the tissue out on the slide to ensure that it is lying flat and easily visible.

iv. Tissue should be analyzed immediately (*see* Note 55).

B. Large tissue (*e.g.* brain, TG)

i. Prepare gelatin solution, vortex, and place it in a 37 °C water bath until fully dissolved. Keep gelatin warm while not in use.

ii. Pour enough gelatin into a base mold to cover the bottom of the mold. Allow the gelatin to solidify at RT.

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50. DIA-Umpire *(*14*)* can be run either via GUI (housed in the FragPipe software program *(*68*)*) or via command line interface (CLI). We prefer to run via CLI, as this minimizes error by using a single parameter file for all analyses. Alternatively, a parameter file can also be loaded into the GUI.

51. For file conversion we use msConvert *(*69*)*

52. For neuropeptide analyses, we have found it best to use the default parameters.

53. It may be helpful to label dissection dishes with the orientation of the tissues to avoid uncertainties after the tissue is dissected.

54. Delicate tissue such as the PO can be transferred to the water by either carefully folding the tissue into quarters or by holding the tissue at either end with separate sets of tweezers.

55. To prevent degradation, a Denator heat stabilizer can be implemented immediately after desalting. However, caution must be taken to avoid melting delicate tissue. For best results, slides should be analyzed as soon as possible. During storage, slides can be wrapped in tin foil to protect them from damage. When removing the slides from the freezer, water will condense on the slides, which can cause diffusion, due to the major temperature change. Place them in a desiccator to minimize this effect.

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iii. Collect the tissue and desalt as described above for small tissue.

iv. Place the tissue on top of the gelatin layer, making note of the tissue orientation. Fill the mold with warm gelatin. The tissue will float toward the top. Reorient the tissue if necessary to make sure it is centered in the mold.

v. Immediately place the mold with tissue in dry ice to flash freeze it (*see* Note 56).

vi. Store tissue at −80 °C until use.

vii. Cross-section tissue using a cryostat.

1. Remove tissue-embedded gelatin block from base mold and trim excess gelatin with a clean razor blade.
2. Place small droplet of water on cryostat chuck and place tissue block on top. Surround the rest of the block with water, making sure to not get any below the chuck.
3. Keep the block/chuck in the cryostat to allow the water to freeze, attaching the tissue block on the chuck (approximately 15 min).
4. Attach the chuck to cryostat and align tissue block so that even slices can be made through tissue.
5. Obtain several 8- to 16-μm sections from throughout the tissue (*see* Note 57). Thaw-mount each section to a glass slide by warming the glass slide slightly and placing it directly above section. Section will adhere to slide.
6. Store slides at −80 °C until ready for analysis (*see* Note 55).

C. Complementary detection using formalin and alternative tissue wash

i. Dry down the tissue slide in a vacuum chamber with desiccant for 5 min.

ii. Prepare a petri dish with 10% NBF, enough volume to fully cover the slide.

56.It is common for slices to fold, tear, or become distorted during the process of thaw mounting. Once the slice is on the slide, thoroughly check it to ensure the integrity of the tissue.

57.Tissue thickness can be decided based on the purpose of the experiment. A recent publication (70) discussed the differences of signal intensity resulted from various slice thickness.

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iii. Submerge the tissue slide into the petri dish for 3 s with tissue facing up.

iv. Remove the slide, incubate, and dry at RT for 2 h.

v. Apply matrix (see following section) and store slides in a desiccator box at –80 °C until imaging experiment.

2. Image Acquisition (MALDI-MS)

A. Matrix Application via Sprayer (*see* Note 58).

i. Spot a peptide calibrant on the tissue slide near the tissue slice.

ii. Position the slide in the sprayer.

iii. Turn on matrix sprayer (we use TM-Sprayer, all subsequent experimental details refer to that) and solvent syringe pump, setting desired solvent flow rate.

iv. Set pressurized air to 10 PSI.

v. Open program and set temperature to 80 °C for DHB (*see* Notes 59 and 31).

vi. Set the desired method (*see* Note 60).

vii. Switch the injection loop to "Load" and load matrix using a syringe pump. Switch the injection loop to "Spray" and ensure that matrix is being sprayed. It may take several minutes for matrix to reach the spray nozzle.

viii. Click "Start" on the Sprayer software (*see* Notes 61 and 62).

ix. When method has finished, flush the matrix loop with 5 ml of solvent each for three times and set the temperature of the sprayer to 30 °C. When the sprayer temperature has lowered to this temperature, turn off sprayer, syringe pump, pressurized air, and software.

B. Analysis on MALDI-LTQ-Orbitrap

i. Insert one or two slides into MALDI imaging plate.

58. There are several methods for applying matrix to a sample, including airbrush, automated sprayer, inkjet printer, sublimation, etc. The method described here utilizes an automated sprayer (*e.g.* TM-Sprayer) which has been found to be the most reproducible (*71*).

59. The sprayer temperature should be high enough to evaporate the matrix/solvent mixture. For the TM-Sprayer, turn up the temperature slowly until you hear a "puffing" noise. Reduce the temperature by 5 °C for the final method.

60. Parameters to consider when choosing a method include the number of times the sprayer passes over and coats the slide, the drying time between coats, the direction the sprayer moves in to coat the slide (*i.e.*, horizontal or vertical), and the system flow rate. Our preferred method includes 12 passes with a 30 s dry time between passes, and the orientation alternating between horizontal and vertical with each pass to provide an even coating at a flow rate of 0.1 mL/min. Methods should be optimized to the peptide group of interest and matrix to minimize diffusion while increasing peptide extraction.

61. The matrix loop only has a finite volume it can hold, and so it may be necessary to reload it with matrix during the course of the method. If this is necessary, wait until the sprayer is done with its current pass, switch the injection loop to "Load," load matrix, and switch the injection loop back to "Spray" before it starts the next pass.

62. After clicking "Start," the system may say the temperature is unstable due to the sensitivity of the system. Since the temperature will never fully stabilize, click "Start Now" to start the method.

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ii. Place plate face-down in scanner, and scan image of entire plate. Ensure that the white crosses in the corners of the plate are clearly visible in the scanned image; otherwise, the image cannot be aligned to the plate in the instrument.

iii. Attach the backing plate to the MALDI imaging plate and insert the plate set into the instrument.

iv. Once the plate has been inserted, click on the "Tissue Imaging" tab of the MALDI Source page and check the box titled "Use tissue imaging feature."

v. Under "Position File," check "Import Image" and upload the scanned image of the MALDI plate (*see* Note 63)

vi. Enter the appropriate raster size (*see* Note 64) and ensure that the size "rectangular" is selected. (Rectangles are the preferred shape for methods.)

vii. Select "View Plate," and click on the square selection tool. Select the area of the tissue. Close the "View Plate" window and save the position file (*see* Notes 65–67).

viii. Click on the "Control" tab and shoot the laser at an area of matrix. Adjust the laser energy as needed to obtain sufficient signal (with our instrument on the order of 1E7).

ix. Open the software setup (Thermo Xcalibur Sequence Setup), and create a new sequence as previously described. For the position, copy and paste the directory of the position file for each tissue into its respective row.

x. Select each line to be run and press the "Run Sequence" button.

C. Analysis with Bruker rapifleX

i. Mark the corner of the ITO tissue slide with a white-out pen for later instrumental teaching and calibration.

ii. Insert one or two slides into MALDI imaging plate.

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[63]. The MALDI-Orbitrap is also capable of scanning the whole plate or each individual slide at varying image qualities. Scanning the plate with an external system only takes a few minutes, while scanning one slide at "normal" resolution takes ~25 minutes. For other instruments, images need to be scanned by an external scanner.

[64]. Raster size determines the spatial resolution of the tissue being imaged. While small raster sizes provide better image resolution, they can drastically increase the analysis time of the instrument. The MALDI-Orbitrap allows for a raster size of 75 microns without oversampling, but other MALDI instruments boast spatial resolution down to a few microns.

[65]. After selecting the final area of interest, saving this imaging in the "View Plate" window allows for easier image overlay during data processing.

[67]. It is good practice to include a small amount of area outside of the tissue when selecting the area to be analyzed in order for it to be used as a means to distinguish signal from random noise.

iii. Place plate with tissue facing down in scanner, and scan image of entire plate. Ensure that the white marks at the corners of the slide are clearly visible in the scanned image.

iv. Insert the imaging plate into the rapifleX.

v. Operate the instrument through the flexImaging and flexControl interface.

vi. In flexControl, select the right geometry (We use MTP Slide Adapter II).

vii. Select desired method for the neuropeptide imaging experiment. Define the laser energy based upon the matrix used (*see* Note 31).

viii. Under flexControl "Calibration", calibrate the desired mass range using the calibrant spotted to the slide.

ix. Follow the steps suggested in the flexImaging interface for imaging run. Load the scanned image to the program and teach the program the orientation of the slide and position the sample carrier.

x. Define the imaging measurement regions by circling around the tissues.

xi. Start automatic run.

D. Data Analysis (See Fig. 4 for example MS images.)

i. ImageQuest (For Thermo instruments)

1. Open raw data in ImageQuest.
2. Click the "New data set" icon and type in the mass of interest and the tolerance window with the "Base Peak" plot type. Under plot type, select the desired normalization, if any (*see* Note 68). Click "OK."
3. To save or export individual images, select the image, and click "Copy" in the Edit tab. Paste the image in the desired location (*see* Note 69).

ii. MSiReader (*see* Note 70) *(17)*

1. Open the raw data in MSiReader using the appropriate open-access format (*see* Note 71)

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68. Typically, normalization is performed in reference to the total ion current (TIC), in which each mass spectrum is divided by its TIC. This ensures that all spectra have the same integrated area under their curves. Other normalization strategies are emerging *(72)*, but TIC continues to be the most widely used.

69. When comparing multiple images, it is often helpful to set them to the same intensity scale. This can be done by entering the Min and Max Plot Values in the "Scale" tab.

70. MSiReader *(17)* is a freely-available, open-access software that can be downloaded from http://www.msireader.com/.

71. To convert to imzML in ImageQuest, click File, Export, imzML. Either all peaks or centroids only can be exported.

2. Enter an *m/z* value from ImageQuest that displays a clear distribution in the tissue.

3. Enter the appropriate parameters for the m/z window, normalization (*see* Note 68), and color map.

4. Use the image overlay feature to upload a scanned image of the tissue and align the image to the MS image of the tissue. Set the transparency to around 50% to ensure both optical and MS images are clearly visible.

5. Generate an image of each neuropeptide signal of interest from a database using "generate an image for each peak in a list" button.

6. Manually examine images for detected neuropeptides and distinct spatial distributions.

7. Search for novel neuropeptides and m/z values outside of the database by using the peak finding tool.

iii. 3D image generation (Image J (38))

1. Open grayscale images of consecutive tissue slices with the same m/z value (saved from ImageQuest).

2. Align the images using the functions under "image."

3. Combine the images into a three-dimensional stack using the "images to stack" button.

4. View the three-dimensional image by clicking "image," "stacks," and then "3D project." Viewing parameters can be adjusted in the 3D projection window.

iv. flexAnalysis (only for imaging file resulted from Bruker rapifleX)

1. In flexImaging, load the data by selecting an existing imaging file (.mis file).

2. In the Spectrum Display window, click the desired peak to look at the distribution of that peak.

3. Right click the highlighted area on the image for certain spectra and select "view spectra in flexAnalysis" to examine the spectra of individual peak in flexAnalysis.

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4. Right click the spectra under Spectrum Display window to export the mass list in .csv format and analyze the data in depth using excel.

5. Save the image of distribution of certain spectra by exporting the image under "File, Export".

6. flexImaging files can also be exported to .img format and be analyzed using MSiReader

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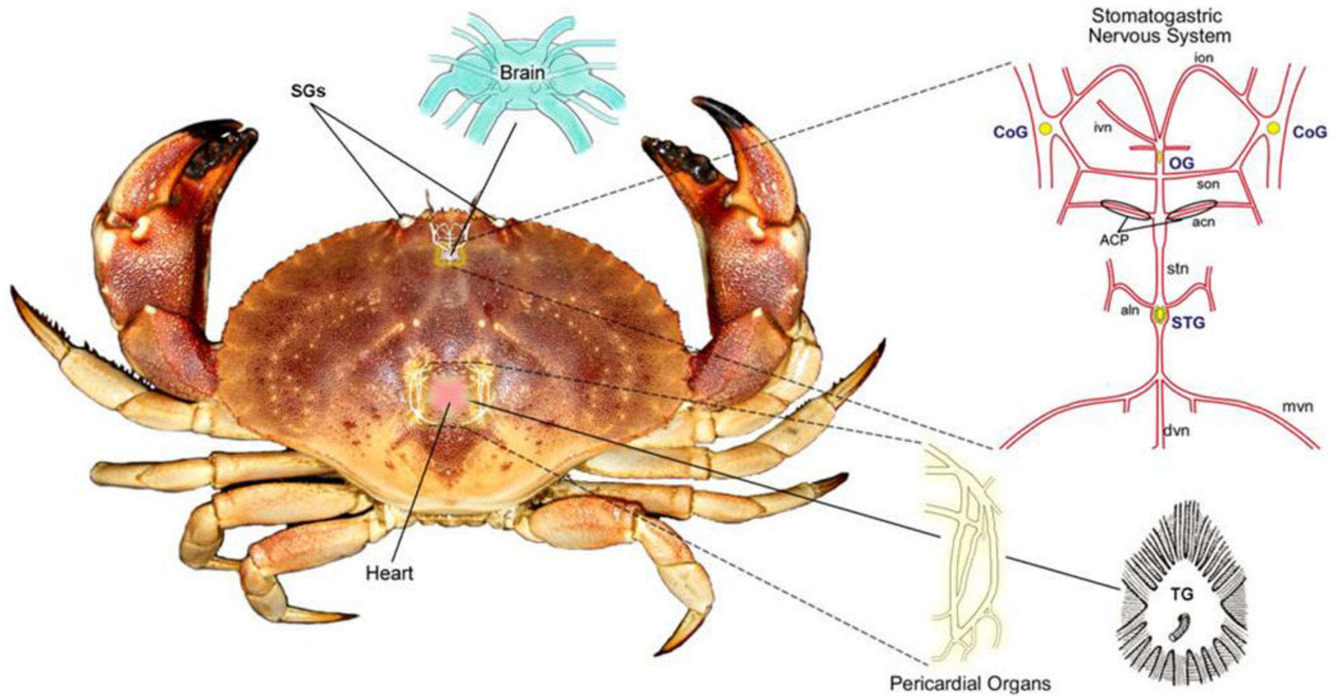

Figure 1.

Schematic diagram of the location of tissues in *Cancer borealis* that are used for peptidomic studies, including the brain, CoGs, OG, STG, TG, POs, and SGs. The location of the heart is also indicated, which is important for probe placement in microdialysis studies. (Reproduced from ref. (20) with permission.)

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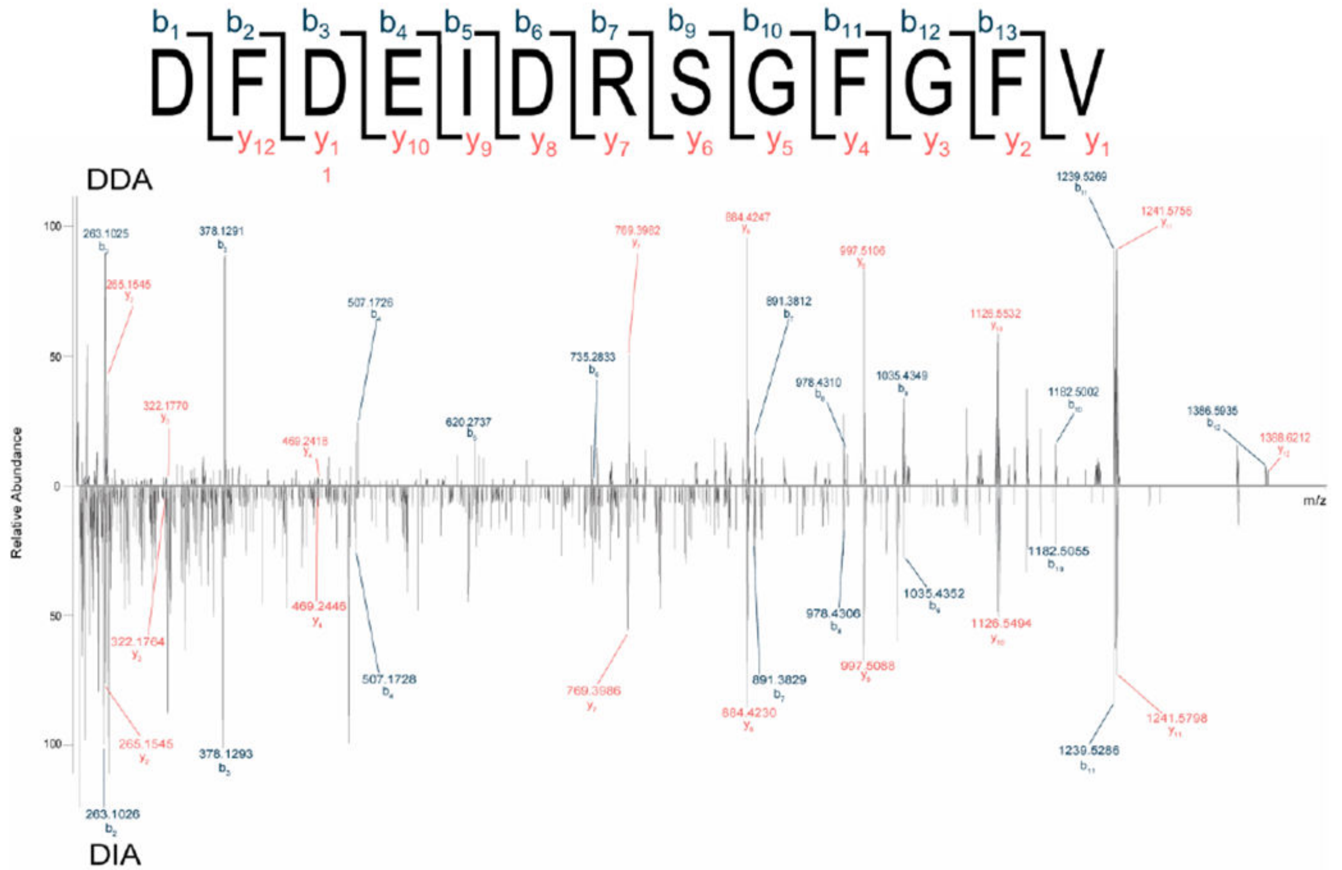

Figure 2.

Annotated spectra from both DDA and DIA experiments with the same neuropeptide identification, illustrating the increased complexity of DIA spectral analysis.

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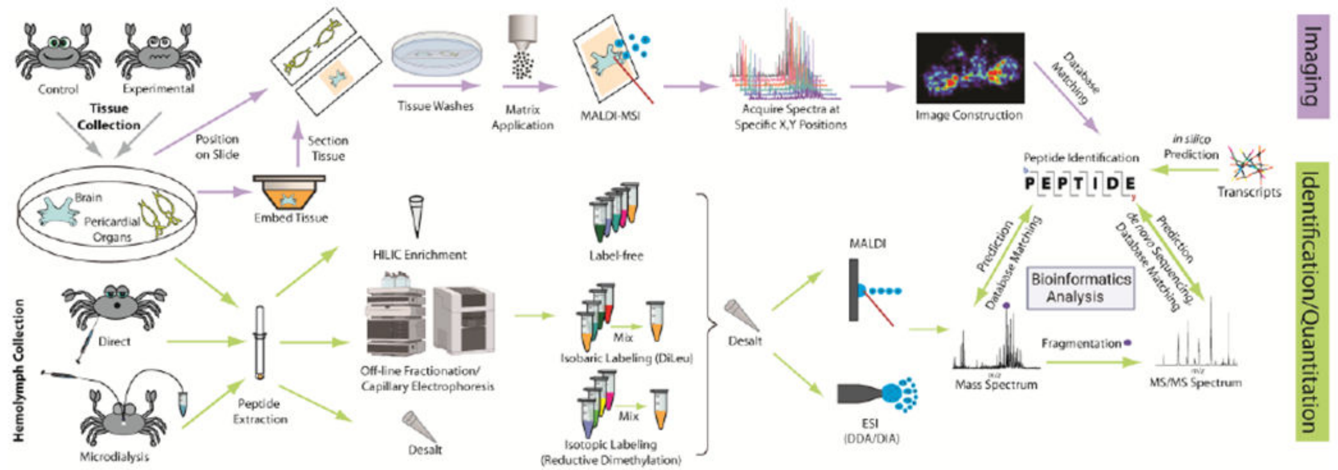

Figure 3.

Workflow indicating the steps for sample collection, preparation, and data acquisition and analysis. The workflow contains steps for analysis of tissues, crude hemolymph, and microdialysis samples and describes the processes for both imaging and quantitation. For relative quantitation, duplex formaldehyde labeling is indicated, with CH_2O indicating nonisotopic formaldehyde and CD_2O indicating deuterated formaldehyde used.

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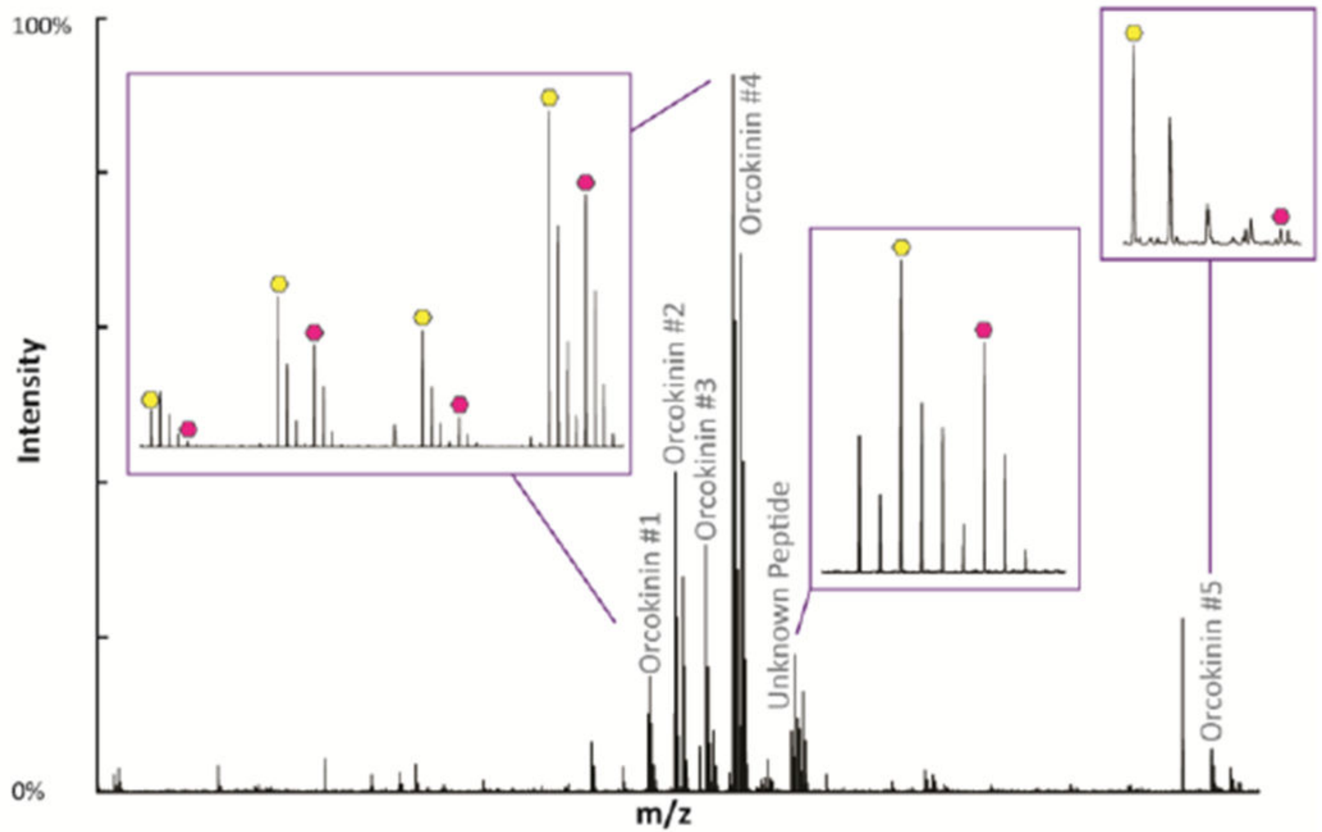

Figure 4.

Example spectrum of results obtained from tissue analysis of blue crab, *Callinectes sapidus* brain using a MALDI-LTQ-Orbitrap XL mass spectrometry. Neuropeptides from the Orcokinin family are indicated, as well as an unknown peptide. Enlarged images show the mass shift between light and heavy-labeled peptides, which enables relative quantitation.

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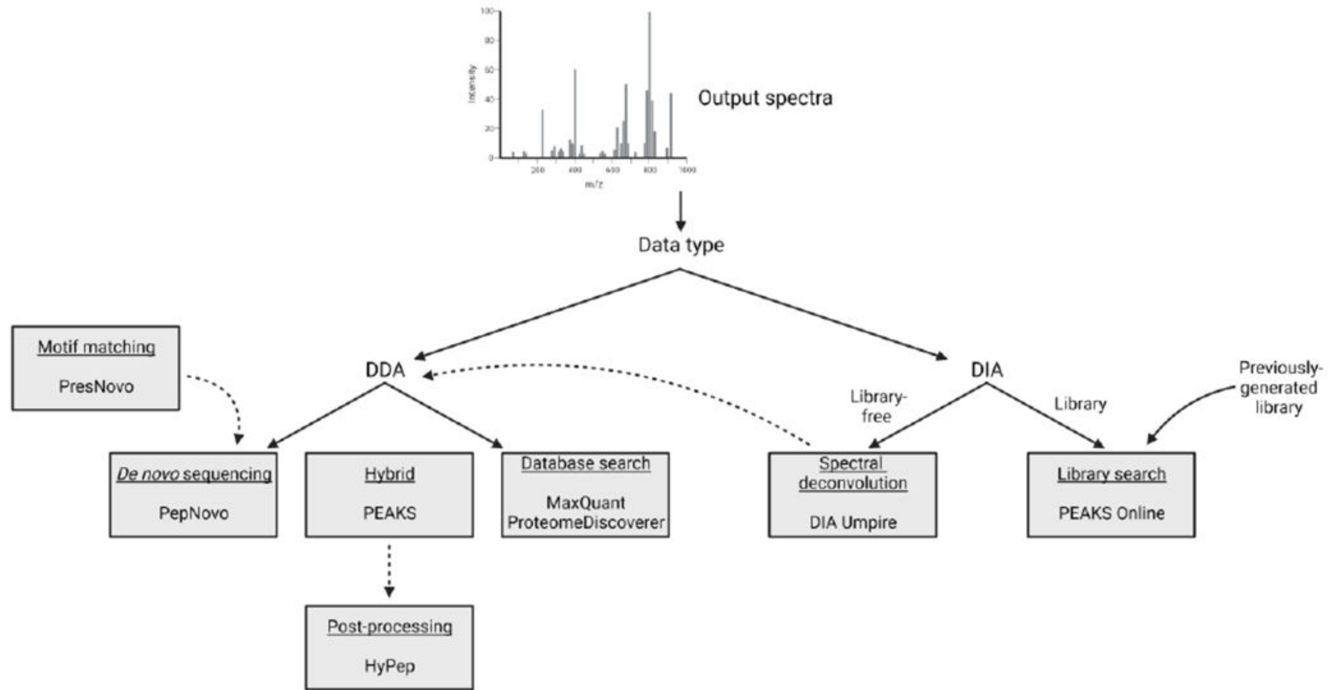

Figure 5.

Computational workflow for output ESI data. DDA spectra can undergo *de novo* sequencing, database searching, or a hybrid of both approaches. DIA spectra can be interpreted through the use of a spectral library, or library-free analysis can be used for spectral deconvolution, followed by DDA search of output pseudospectra.

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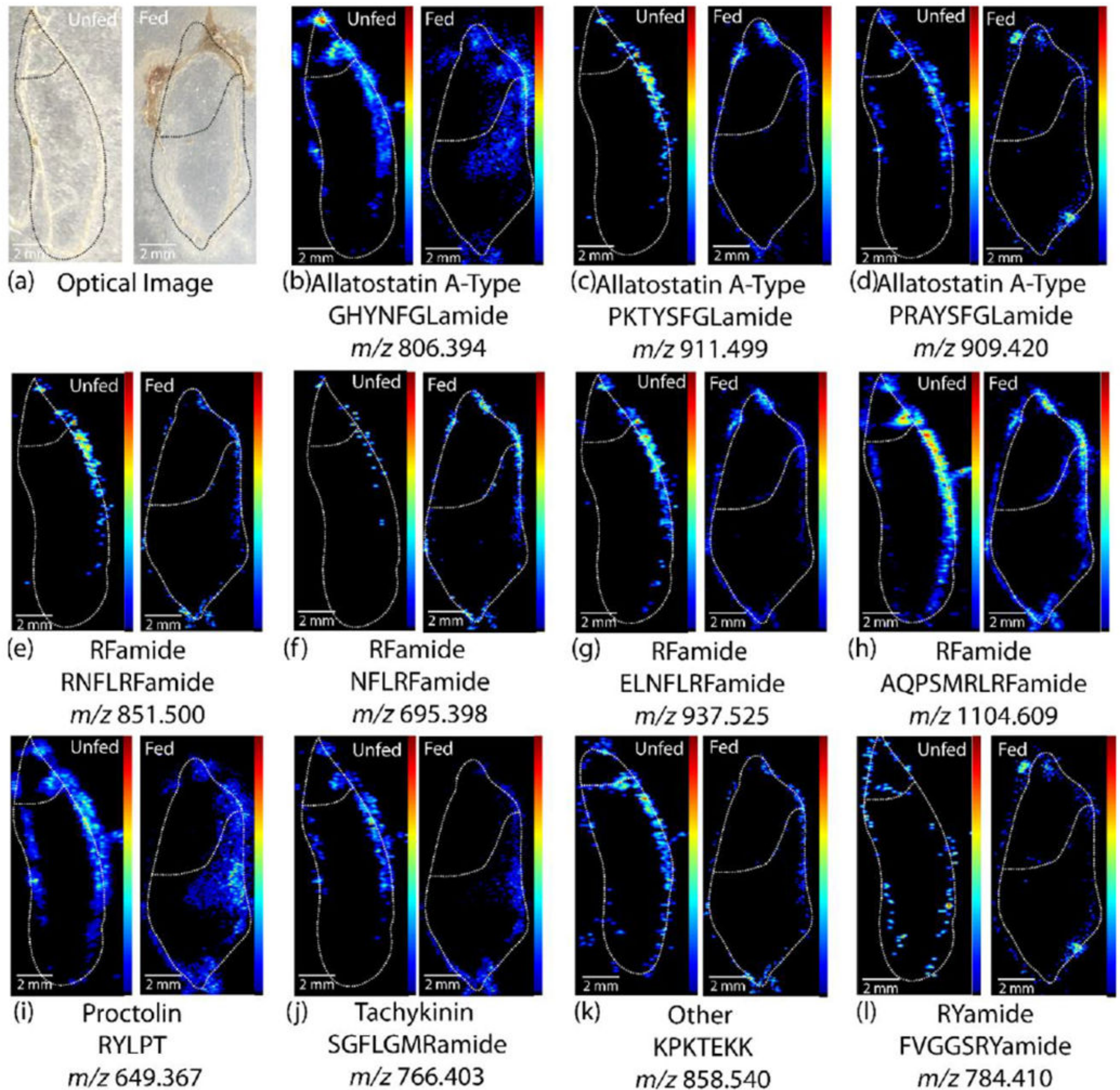

Figure 6.

Example MS images of a Jonah crab, *Cancer borealis*, PO. Images were obtained on a MALDI-LTQ-Orbitrap XL and showed the spatial distribution of eleven selected *m/z* values. All images were normalized to the TIC and the optical image of the tissue was also shown in the figure for reference. (Reprinted with permission from ref. (*32*). Copyright 2021 American Chemical Society.)